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International standards and international reference preparations

Amphotericin B, vancomycin, capreomycin, cefalotin, demethylchlortetracycline, gentamycin, gramicidin S, kanamycin and kanamycin B, lincomycin, lymecycline, methacycline, paromomycin, rifamycin SV, ristocetin and ristocetin B, spiramycin, and triacetyloleandomycin

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Each of the preparations described here was obtained and evaluated at the request of a WHO Expert Committee on Biological Standardization. Unless otherwise stated, a standard procedure was used to distribute the material into individual ampoules. The procedure was as follows. Upon receipt by the National Institute for Medical Research (NIMR), London, materials were stored temporarily in the dark at a temperature of −10°C or lower, and protected from moisture. At a convenient time they were brought back to room temperature, mixed, and distributed into individual neutral glass ampoules so that each ampoule contained 50–100 mg of powder. If it was known that the material was light-sensitive non-actinic glass ampoules were used. After exhaustive drying in vacuum over phosphorus(V) oxide, the ampoules were either constricted (up to 1963) or fitted with capillary leak plugs, dried for a further period under the same conditions, filled with dry nitrogen, and sealed by fusion of the glass. The total drying period varied from 8 to 38 days according to the nature of the material. After they had been tested for leaks, the ampoules were stored in the dark at −20°C.

AMPHOTERICIN B

The sample of amphotericin B was obtained in 1958 by the generosity of E. R. Squibb and Son, New Brunswick, N.J., USA, and through the good offices of Dr H. Welch, US Food and Drug Administration (FDA). The following description of the sample was supplied by Dr A. F. Langlykke of E. R. Squibb and Son.

weight	100 g.
lot no.	CA/136/A
form	crystalline
chemical assay	1 000 μg/mg of amphotericin B
biological assay	940 "μg" [1]/mg ± 5%
specific rotation, $[\alpha]_D^{25}$	−420°
amphotericin A:	nondetectable by measuring extinction at 405 nm
storage:	at −20°C or lower suggested

Following confirmation that this material had adequate stability at −20°C, it was established in 1959 as the International Reference Preparation of Amphotericin B, and the International Unit was defined in 1962 as the activity contained in 0.001064 mg of the International Reference Preparation, corresponding to a potency of 940 IU/mg.

Experience in the use of this preparation over several years showed that it was suitable to serve as an international standard. It was therefore established in 1963 as the International Standard for Amphotericin B; the definition of the international unit was not changed.

References

WHO Expert Committee on Biological Standardization (1959,[3] 1960, 1963, 1964a).

[1] WHO International Laboratory for Biological Standardization, National Institute for Medical Research, London, England.

[2] When μg is placed between quotation marks, thus: " μg ", it refers to a certain amount of activity (potency) and not to its accepted usage as a unit of weight.

[3] Authorization.

VANCOMYCIN

A suitable sample of vancomycin sulfate was obtained by the generosity of Eli Lilly and Company, Indianapolis, Ind., USA, and through the good offices of Dr H. Welch of the FDA. The following description was supplied by Dr W. B. Fortune of Eli Lilly and Company.

weight	100 g	
lot no.	302-174AD-191B	
microbiological assay	955 base units/mg (undried)	
chemical assay	870 base units/mg (undried), based on an enzymatic glucose reaction	
water (Karl Fischer method)	6.23%	
ash	0.78%	dry basis
carbon	51.35%	dry basis
hydrogen	5.35%	dry basis
nitrogen	9.16%	dry basis
sulfur	1.05%	dry basis
amino nitrogen	2.43%	dry basis
total chlorine	4.68%	dry basis
ionic chlorine	nil	

percentage purity by chelation with Cu^{++} not less than 95% (dry basis)

This material was established in 1959 as the International Reference Preparation of Vancomycin. The moisture content of the material distributed into ampoules, measured as the loss in weight at 56.5°C over phosphorus(V) oxide at a pressure of 0.01 mm Hg, was found to be 1.08% w/w. When exposed to the atmosphere at a relative humidity of 47%, the material in the ampoules increased in weight by approximately 0.5% in 5 min.

The potency of the material in the ampoules corrected for the change in moisture content was therefore 1 007 units/mg, and the International Unit was defined in 1962 as the activity contained in 0.000993 mg of the International Reference Preparation, corresponding to a potency of 1 007 IU/mg.

Further studies showed that this material was suitable to serve as an international standard and it was established in 1963 as the International Standard for Vancomycin; the definition of the international unit was not changed.

Stability

Accelerated degradation studies of the International Standard for Vancomycin indicated that the loss of activity to be expected in 10 years at −20°C is less than 1.0%.

References

WHO Expert Committee on Biological Standardization (1959,[1] 1960, 1963, 1964a).

CAPREOMYCIN

By the generosity of Dista Products Ltd, Speke, England, and through the good offices of Dr H. Tozer of that company, 400 g of capreomycin sulfate was obtained in 1967. The following data were supplied by the manufacturer.

reference no. of sample	595AD-116
microbiological assay	852 units/mg
loss on drying for 4 h at 100°C and 5 mm Hg over phosphorus(V) oxide	7.31% w/w
microbiological assay after drying as above	920 units of base activity/mg
water (Karl Fischer method)	5.98%
methanol	2.37%
sulfate (undried)	20.45%
ash	0.14%
calcium	0.02%
heavy metal	nil
pH	5.0
LD_{50} (mice)	228×10^3 units/kg
ratio of capreomycin 1A to 1B	1.16
capreomycin II	1.5%

The potency values reported by the manufacturer were obtained by biological assay, turbidimetrically using *Klebsiella pneumoniae*, and by assay in terms of a Lilly Indianapolis reference standard lot AC-4 RR (P-87562) with an assigned potency of 750 units/mg (June 1966). The following history of the Lilly reference standard and the definition of the unit was provided by the manufacturer.

[1] Authorization.

The first standard for capreomycin was assigned a potency in micrograms of capreomycin base per milligram at a time when approximate molecular weight information became known. This was before it was known that capreomycin consisted of four components. Since then, succeeding standards have been equated on a basis of the microbiological turbidimetric assay using *Klebsiella pneumoniae* and this eventually gave rise to P.87562 with a potency of 750 μg/mg. However, with the knowledge that capreomycin is composed of four components which are not all equal in specific activity and which have different molecular weights, it was ambiguous to express potency as micrograms per milligram. The standard P.87562 was then assigned a potency of 750 units/mg and the response of this standard with a value of 750 units/mg in the turbidimetric assay using *Klebsiella pneumoniae* constitutes the definition of a capreomycin unit.

The material in the sealed ampoules showed no significant loss of weight when heated at 100°C over phosphorus(V) oxide at a pressure of 0.04 mm Hg for 4 h. When it was exposed to the atmosphere with a relative humidity of 66% it increased in weight by approximately 1.0% in 5 min and by 6.0% in 30 min. It was established in 1967 as the International Reference Preparation of Capreomycin and the International Unit was defined as the activity contained in 0.001087 mg of the International Reference Preparation, corresponding to a potency of 920 IU/mg.

Stability

Samples of this preparation lost 5.6% of their biological activity after storage at 56°C for 1 year.

References

WHO Expert Committee on Biological Standardization (1964b,[1] 1968).

CEFALOTIN

A suitable sample (50 g) of cefalotin, sodium salt, was obtained in 1964 by the generosity of Eli Lilly and Company, Indianapolis, Ind., USA, and through the good offices of Dr W. W. Wright of the FDA. The following data were supplied by the manufacturer.

lot no.	293-453AD-126AB
assayed by agreement with the FDA (iodometric assay of sample dried *in vacuo* at 60°C)	938 μg/mg
microbiological assay (undried)	947 "μg"/mg
$[\alpha]_D^{25}$ (5.0% aqueous solution)	+128.1°
UV ratio, 237 nm/265 nm	1.64
loss on drying (3 h *in vacuo* at 60°C)	0.19%
pH of 30 mg/ml solution in water	5.35

This sample was taken from the same batch used for the standard preparation of the FDA, which had an assigned potency of 938 " μg "/mg when dried.

After distribution of the material into ampoules, no significant loss in weight was observed when samples were heated at 56°C in vacuum (< 0.02 mm Hg) over phosphorus(V) oxide. The potency of the material in the ampoules was therefore 938 " μg "/mg. When exposed to the atmosphere at a relative humidity of 50%, the material in the ampoules increased in weight by approximately 0.2% in 30 min.

The material was established in 1965 as the International Reference Preparation of Cefalotin.

Assays carried out in the laboratories of the FDA, Washington, DC, USA, and the NIMR, London, of the International Reference Preparation in terms of the FDA standard gave the following results.

Laboratory	*Day*	*Assay*	*Potency (μg/mg)*	*95% confidence limits (μg/mg)*
FDA	1	1	938	912–968
		2	926	871–982
	2	3	942	913–973
		4	923	903–944
	3	5	934	903–965
NIMR	1	1	927.2	915.1–939.0
		2	909.9	897.7–922.2
	2	1	915.5	904.3–926.9
		2	972.2	960.9–983.4
	3	1	936.2	923.4–949.1
		2	934.7	923.9–945.5

These results were consistent with the expected potency of 938 " μg "/mg and the International Unit for Cefalotin was defined in 1965 as the activity

[1] Authorization.

contained in 0.0010661 mg of the International Reference Preparation, corresponding to a potency of 938 IU/mg.

Stability

A sample of the material contained in ampoules and stored for 1 year at 37°C was assayed. No significant loss of potency had occurred.

References

WHO Expert Committee on Biological Standardization (1964b,[1] 1966).

DEMETHYLCHLORTETRACYCLINE

Suitable material was obtained in 1962 by the generosity of Cyanamid of Great Britain Ltd, and through the good offices of Mr W. P. Jones of that company. It consisted of 200 g of demethylchlortetracycline hydrochloride (7117B-53) that had been specially prepared on a laboratory scale. The sample was reported by the manufacturer to be "100% pure" on a dry weight basis (drying for 3 h at 60°C at a pressure of less than 5 mm Hg). Chromatographic analysis had shown the presence of traces only of homologous compounds estimated to represent less than 0.1% of the total weight. It was established in 1962 as the International Reference Preparation of Demethylchlortetracycline and the International Unit was defined as the activity contained in 0.001 mg of the International Reference Preparation corresponding to a potency of 1 000 IU/mg.

Stability

Accelerated degradation studies of this preparation have demonstrated no significant loss of activity after storage of ampoules at 37°C for 2 years.

References

WHO Expert Committee on Biological Standardization (1963).[2]

GENTAMYCIN

Suitable material obtained in 1967 by the generosity of Schering, USA, consisted of 150 g of gentamycin sulfate, and was part of the batch GMC 4J-1-4C used to provide the working standard of the FDA.

The following data were supplied by the manufacturer.

loss on drying	8.04% (3 h at 110°C and a pressure of 5 mm Hg or less)
$[\alpha]_D$ (dried; in 1% aqueous solution)	115°
sulfate	present [32.29% w/w when later determined in the NIMR laboratories]
identity (IR)	satisfactory
solubility	satisfactory
potency, when dried for 3 h at 110° and a pressure of 5 mm Hg or less	641 "μg"/mg
composition	
by radioisotope method	64% C_1, 36% C_2
bioassay technique	24.7% C_1, 38.9% C_{1a}, 36.4% C_2

The 64% C_1 as determined by the radioisotope method comprises the two components C_1 and C_{1a} determined separately by the bioassay technique.

After distribution into ampoules the contents showed no significant loss in weight when dried for 3 h at 110°C and at a pressure of less than 5 mm Hg. When exposed to the atmosphere at a relative humidity of 50%, the material in the ampoules increased in weight by approximately 2.5% in 2 min and 3.8% in 10 min.

The material was assayed against the working standard of the FDA, with which it should be identical and which had a defined potency of 641 "μg"/mg (dried). Assays were carried out in the laboratories of the FDA, Washington, DC, through the good offices of Dr W. W. Wright and also at the NIMR, London, with the following results (weighted mean potency ratio, i.e., ratio between the proposed International Reference Preparation and the FDA working standard).

[1] Authorization.

[2] Including the authorization.

Laboratory	*Potency ratio*	*95% confidence limits*
FDA (6 assays)	1.0003	0.98207–1.0189
NIMR		
(4 assays, day 1)	1.0304	1.0141–1.0469
(4 assays, day 2)	0.9915	0.973–1.0103

Although the confidence limits of all the assays did not include 1.000, the apparent difference between the two lots of material was very small and may have arisen from the technique of drying used for the FDA working standard. With respect to the NIMR values, on day 1 the vacuum obtained during drying in the vacuum oven was only just that required, i.e. 5 mm Hg, whereas on day 2 drying was effected in a " drying pistol " at a pressure of 0.05 mm Hg. It was therefore concluded that no difference had been introduced by the processing of these two different samples of the same batch of material, other than that of the content of volatile substance. The potency of the proposed international reference preparation would therefore be 641 units/mg if the unit were made equal to the " microgram of activity " of the current FDA working standard.

The International Reference Preparation of Gentamycin was established in 1968 and the International Unit was defined in 1968 as the activity contained in 0.00156 mg of the International Reference Preparation, corresponding to a potency of 641 IU/mg.

References

WHO Expert Committee on Biological Standardization (1967,[1] 1969).

GRAMICIDIN S

In 1962, 90 ampoules each containing approximately 50 mg of a sample of gramicidin S were received through the generosity of the Government of the USSR. The material had been specially prepared and purified at the Institute of Antibiotics, Academy of Medical Sciences, Moscow, under the direction of Professor G. F. Gause; it was characterized and distributed into ampoules by Dr L. S. Ogloblina and Dr L. M. Jacobson, who submitted the following report.

Purity before distribution on dry weight basis, 100%.
Moisture content, 4% w/w.
The sample was distributed in 50-mg amounts into ampoules and dried for 9 days over phosphorus(V) oxide in vacuum; the ampoules were filled with dry nitrogen and sealed. The seals of the ampoules were checked to detect fissures and breakage, after which the ampoules were stored at 4°C until dispatch to London.
Moisture content after drying, 0.135% w/w.
Light absorption, 0.1 mg/ml in absolute ethanol showed maximum extinction at 250 nm and 270 nm.
$[\alpha]_D^{20}$ in 96% ethanol, 290°.
Solubility in 96% ethanol at 20°C, 10.5%.
pH of 10 mg in 1 ml of a solution of 96% ethanol and 10 parts of water, 6.6.
Estimated purity, 99.86%.

It was reported by Dr Ogloblina that the unit of activity used for gramicidin S was equated to the microgram of pure gramicidin S and therefore the potency of the material in the ampoules was 998 units/mg.

The International Reference Preparation of Gramicidin S was established in 1962 and the International Unit defined in 1963 as the activity contained in 0.001002 mg of the International Reference Preparation, corresponding to a potency of 998 IU/mg.

References

WHO Expert Committee on Biological Standardization (1961,[1] 1963, 1964a).

KANAMYCIN

A suitable sample was obtained in 1958 by the generosity of Bristol Laboratories Inc., Syracuse, N.Y., USA, and through the good offices of Dr H. Welch, of the FDA. The following description of the sample, which was in the form of kanamycin sulfate, was supplied by Dr H. A. Frediani of Bristol Laboratories.

[1] Authorization.

weight	100 g.
lot no.	E 8651
form	crystalline
potency (without drying)	770 "μg"/mg
moisture (Karl Fischer method)	5.5%
$[\alpha]_D$	+126°
sulfate (anhydrous basis)	17.2%
sulfated ash	0.2%
kanamycin A content (paper strip method)	99.9%

After the material had been distributed into ampoules, the moisture content was measured as weight lost at 56.5°C over phosphorus(V) oxide at a pressure of 0.01 mm Hg; it was found to be 0.37% w/w. When exposed to the atmosphere at a relative humidity of 47%, the material in the ampoules increased in weight by approximately 0.1% in 10 min. The water content is difficult to determine precisely by the Karl Fischer method on quantities as small as 50 mg (i.e., the average content of an ampoule) and the value obtained by measurement of weight loss was considered the more reliable and was used to calculate the change in potency that had resulted from drying. The potency of the material in the ampoules corrected for the change in moisture content was therefore 813 units/mg.

The material was established in 1959 as the International Reference Preparation of Kanamycin and the International Unit was defined in 1962 as the activity contained in 0.001232 mg of the International Reference Preparation, corresponding to a potency of 812 IU/mg.

Stability

Samples of the International Reference Preparation of Kanamycin stored at 37°C for 4 years showed no significant loss of biological activity.

References

WHO Expert Committee on Biological Standardization (1959,[1] 1960, 1963).

KANAMYCIN B

There was a need for international reference preparations of kanamycin A and B since material used clinically contained both components, kanamycin A being the major component. An international reference preparation of kanamycin B would facilitate the control of the concentration of kanamycin B in kanamycin intended for clinical use. This is important because of the greater toxicity of kanamycin B.

The International Reference Preparation of Kanamycin had been reported to contain 99.9% of kanamycin A when examined by paper chromatography, and it appeared that this material was suitable to serve as an international reference material of kanamycin A.

By the generosity of Bristol Laboratories, Syracuse, N.Y., USA, and through the good offices of Dr Frediani of that company, 2.5 g of kanamycin B base were obtained in 1964. This sample was part of the same lot (3927 29C-4) provided to the FDA for use as a reference standard of kanamycin B. The following information was supplied by the manufacturer.

potency	100% kanamycin B (*US Pharmacopoeia*)
moisture (Karl Fischer method)	8.3%
ash, sulfated	0.5%
kanamycin A (paper strip method)	<0.1%
sulfate (qualitative)	nil

The 2.5 g (approximately) of kanamycin B base was accurately weighed and dissolved in distilled water so that 1 ml contained exactly 5.00 mg of the preparation; the solution had a pH of 9.25. Accurately measured aliquots of this solution were distributed into ampoules, the volume of the aliquot being such that each ampoule contained 4.67 mg of anhydrous kanamycin B base. Check weighings on 15 ampoules showed that the variation in the size of the aliquots of solution was less than ±0.5%. After freeze-drying the ampoules were processed by the general procedure already described.

Both preparations, i.e., the International Reference Preparation of Kanamycin and the proposed international reference preparation of kanamycin B, had been found by the manufacturer to contain not less than 99.9% of a single component when examined by paper chromatography. The two materials

[1] Authorization.

were further examined in the NIMR laboratory by ion-exclusion chromatography, using a modification of the method of Rothrock et al. (1959) and Maehr & Schaffner (1964), 10-mg quantities being used on columns containing 4.0 ml of an ion-exchange resin.[1] The columns were developed with water (1 ml in 15 min) and aliquots of 0.3 ml collected. The amount of kanamycin in each aliquot was measured by means of a ninhydrin reaction, the extinction of the colour developed being measured at 570 nm. By this method of examination each preparation was found to be free from the presence of the other. The sensitivity of the method was such that the presence of 0.25% of one kanamycin in the other would have been detected.

Using the method of assay of kanamycin B in kanamycin described in the US Pharmacopoeia (US Pharmacopoeial Convention, 1962), a quantity of the International Reference Preparation of Kanamycin was hydrolysed with hydrochloric acid at 100°C and the residual biological activity was measured against the proposed international reference preparation of kanamycin B. The residual activity was equivalent to a concentration of less than 0.5% of kanamycin B in the International Reference Preparation of Kanamycin.

When the proposed international reference preparation of kanamycin B was treated in a similar way the residual activity was equivalent to approximately 10% of that originally present. This loss of activity of kanamycin B on acid hydrolysis is approximately that reported by Dr W. W. Wright (private communication).

This method of estimation of the kanamycin B content, on the basis of residual biological activity after acid hydrolysis, was first described by Wakazawa et al. (1960). They showed that the residual activity could be measured in terms of the original kanamycin B content if a standard preparation of kanamycin B was hydrolysed in parallel with the unknown sample. Since no reference preparation of kanamycin B was available, they suggested that the residual activity should be measured against a kanamycin A standard. This method, however, depends on the relative responses produced by kanamycin A and hydrolysed kanamycin B retaining a constant relationship; the availability of an international reference preparation of kanamycin B should make it unnecessary to attempt to standardize the biological response in this way.

The preparation of kanamycin B described above was established in 1964 as the International Reference Preparation of Kanamycin B. No international unit of activity was defined.

References

WHO Expert Committee on Biological Standardization (1964a,[1] 1964b).

LINCOMYCIN

A suitable sample (100 g) of lincomycin hydrochloride monohydrate was obtained in 1964 by the generosity of the Upjohn Company, Kalamazoo, Mich., USA, and through the good offices of Dr W. W. Wright of the FDA. The following data were supplied by the manufacturer.

lot no.	14121-25
$[\alpha]_D$	+143°
pH (1% aqueous solution)	4.8
water (Karl Fischer method)	3.9% in January 1963 3.8% in December 1963
equivalent weight	450
potency	865 "μg"/mg on an undried basis
lincomycin B content	1.99%

This lot has been examined by phase solubility analysis. Its purity in an acetone–water mixture (93 : 7) was found to be 99.8 ± 0.2%. The result in a mixture of ethyl acetate and ethanol (1 : 1) was 99.7 ± 0.6%.

This sample was taken from the same batch as that used for the standard preparation of the FDA, and had an assigned potency of 865 "μg"/mg, undried. After being distributed into ampoules, the moisture content of the material was determined (in the laboratories of Upjohn and Co., UK, by the Karl Fischer method and found to be 2.08% w/w (mean of 3 determinations: 2.11, 2.07, and 2.08%). The potency of the material corrected for the change in water content from 3.85% w/w to 2.08% w/w was therefore 881 "μg"/mg. The material in the ampoules

[1] Biorad Anion-Exchange Resin AG 1–X 2.

[1] Authorization.

increased in weight by approximately 1.0% w/w in 10 min when exposed to the atmosphere at a relative humidity of 58%.

The preparation was assayed in terms of the FDA standard in the laboratories of the FDA, Washington, DC, and the NIMR, London. The results obtained were as follows.

Laboratory	*Day*	*Assay*	*Potency ("μg"/mg))*	*95% confidence limits ("μg"/mg)*
FDA	1	1	861	836–885
		2	854	816–892
	2	3	883	850–919
		4	833	795–871
	3	5	865	823–912
		6	881	853–912
NIMR				
	1	1	901.9	890.1–912.3
		2	885.6	873.4–897.8
	2	1	894.1	872.4–915.0
		2	875.6	850.8–900.9

These results were consistent with the expected potency of 881 "μg"/mg, and the preparation was established in 1965 as the International Reference Preparation of Lincomycin and the International Unit was defined in 1965 as the activity contained in 0.0011351 mg of the International Reference Preparation, corresponding to a potency of 881 IU/mg.

Stability

After storage of samples of the International Reference Preparation of Lincomycin at 37°C for 1 year no significant change in potency occurred.

References

WHO Expert Committee on Biological Standardization (1964b,[1] 1966).

LYMECYCLINE

The material used was the residue of the British Standard (1963), a specially prepared sample of lymecycline batch 90 B supplied by Carlo Erba SpA, Milan, Italy. The following analytical data describing the sample were provided by the manufacturer.

Microbial assay (turbidimetric assay with *Staphylococcus aureus* ATCC 6538-P (1) 89.2% w/w; (2) 87.9% w/w as tetracycline-L-methylenelysine:

pH (1% solution)	7.9
10% solution in water	completely clear, stable for over 24 h
water (Karl Fischer method)	2.08%
$[\alpha]_D^{20}$ (0.5% in water)	−192°
free tetracycline base	0.14%

Microanalysis (determined on samples left exposed to air until saturated with moisture, correcting the values obtained on the basis of the Karl Fischer value):

theoretical	C, 57.79%; H, 6.35%; N, 9.29%; O, 26.61%
found	C, 57.77%; H, 6.76%; N, 8.96%; O, 26.23%
spectrophotometric purity (on dry weight basis)	99.03%
epimer content	5.7%

The moisture content of the material in the sealed ampoules when measured as loss in weight on drying over phosphorus(V) oxide at 56°C and a pressure of less than 0.05 mm Hg was found to be less than 0.1% w/w. Taking the content of lymecycline of the original material to be 88.5% (mean of 89.2 and 87.9), then the material in the ampoules after drying contained 90.3% of lymecycline. The potency of the British Standard was defined in 1963 as 903 units/mg.

This material was established in 1968 as the International Reference Preparation of Lymecycline and the International Unit defined in 1968 as the activity contained in 0.001107 mg of the International Reference Preparation, corresponding to a potency of 903 IU/mg.

Stability

Accelerated degradation studies carried out on samples of the material in ampoules stored at 56°C and 37°C indicated that the loss in activity of the material stored at −20°C would be less than 1% in 10 years.

References

WHO Expert Committee on Biological Standardization (1968)[2].

[1] Authorization.

[2] Including the authorization.

METHACYCLINE

A suitable sample (50 g) of methacycline hydrochloride, part of lot no. 57522-02 EA (the same lot from which the current FDA working standard for methacycline was taken), was obtained in 1968 by the generosity of Charles Pfizer and Co. Inc., USA, and through the good offices of Dr W. W. Wright of the FDA. The following data were supplied by the manufacturer.

Test	*Pfizer results*	*FDA results*
water (Karl Fischer method)	0.51%	0.6, 0.57 } 0.58%
volatile substances	0.33%	
pH	2.40	2.3, 2.4 } (1% 2.4 aqueous solution)
paper chromatography:		
main band	R_f 0.35 approx.	
thin-layer chromatography:		
main band	R_f 0.1 approx.	
light MP	R_f 0.00 approx.	
light LP	R_f 0.15 approx.	
A (1%, 1 cm) at 345 nm	320.80	
heavy metals	5-10 ppm	
iron	15 ppm	
chlorides	7.37%	
fluoride	8 ppm	
sulfur	35 ppm	
penicillin content	negative	
residue on ignition	0.04%	

After distribution into ampoules a sample of the material was heated over phosphorus(V) oxide, in vacuum at a pressure less than 0.05 mm Hg at 60°C for 4 h; no significant loss in weight was observed. When a sample was exposed to air at a relative humidity of 45% it increased in weight by approximately 0.8% in 4 min, and then by a further 0.15% approximately in 55 min.

The material was assayed against the FDA working standard for methacycline which had an assigned potency of 924 " μg "/mg when dried for 3 h at 60°C and a pressure of 5 mm Hg or less, by the laboratories of the FDA, Washington, DC, and of the NIMR, London. The following results were obtained for the potency ratio, i.e., the ratio between the proposed International Reference Preparation and the FDA working standard. All assays were turbidimetric except those reported by the NIMR for day 1, which were plate-diffusion assays.

Laboratory	*Day*	*Weighing*	*Potency ratio*	*95% confidence limits*
NIMR	1	1	1.038	1.021–1.055
	1	2	0.998	0.986–1.010
	2	3	1.011	0.981–1.041
	3	4	0.997	0.981–1.012
	3	5	0.997	0.986–1.008
	4	6	1.012	0.963–1.063
	4	7	1.004	0.951–1.061
mean (NIMR)			0.9998	0.9918–1.008
FDA	1	1	1.019	0.973–1.069
	1	2	1.000	0.970–1.030
	2	3	1.049	0.997–1.105
	2	4	0.986	0.944–1.031
	3	5	0.985	0.950–1.020
mean (FDA)			1.0076	0.9729–1.043

These results were consistent with the expected potency ratio of 1.000.

The material was established in 1968 as the International Reference Preparation of Methacycline and the International Unit was defined in 1968 as the activity contained in 0.001082 mg of the International Reference Preparation, corresponding to a potency of 924 IU/mg.

References

WHO Expert Committee on Biological Standardization (1968,[1] 1969).

PAROMOMYCIN

A suitable sample (310 g) of paromomycin sulfate was obtained in 1964 by the generosity of Parke, Davis and Co., Detroit, Mich., USA. The following data were supplied by the manufacturer:

batch no.	Rx X7360
potency (microbiological plate assay)	750 "μg"/mg

[1] Authorization.

$[\alpha]_D^{25}$ (dry basis)	+53.29°
loss on drying	7.34%
sulfated ash	0.62%
pH	6.10
sulfate	25.76%
UV and IR curves	satisfactory

This batch was taken from the same batch used for the FDA standard preparation, which had an assigned potency of 750 " μg "/mg when dried for 3 h at 60°C at a pressure of 5 mm Hg or less. After distribution into ampoules no significant loss in weight occurred when the material was heated at 56°C in vacuum at a pressure less than 0.02 mm Hg over phosphorus(V) oxide for 5 h. The potency of the material in the ampoules was therefore 750 "μg"/mg.

When the material from the ampoules was exposed to the atmosphere with a relative humidity of 50% it increased in weight by less than 1.0% in 30 min.

When the material was examined at the NIMR, London, by ion-exclusion chromatography according to the method of Maehr & Schaffner (1965), the separation showed that the sample consisted predominantly of paromomycins A + B (approx. 94%) with small amounts of paromomycin D (approx. 5.5%) and paromomycin C (approx. 0.5%).

The material was established in 1965 as the International Reference Preparation of Paromomycin and was assayed in terms of the FDA standard in the laboratories of the FDA, Washington, DC, and of the NIMR, London. The results obtained were as follows.

Laboratory	*Day*	*Assay*	*Potency ("μg"/mg)*	*95% confidence limits ("μg"/mg)*
FDA	1	1	763	694–842
		2	680	617–739
	2	3	722	686–758
		4	756	712–806
	3	5	694	660–729
		6	740	689–794
NIMR	1	1	761.9	745.6–778.6
		2	777.5	761.1–794.2
	2	3	730.2	715.7–744.9
		4	753.4	731.6–775.8

These results were consistent with the expected potency of 750 "μg"/mg and the International Unit was defined in 1965 as the activity contained in 0.001333 mg of the International Reference Preparation, corresponding to a potency of 750 IU/mg.

Stability

Material from ampoules of the International Reference Preparation of Paromomycin that had been stored for 1 year at 56°C showed no significant loss of activity.

References

WHO Expert Committee on Biological Standardization (1964b,[1] 1966).

RIFAMYCIN SV

A suitable sample (300 g) of the sodium salt of rifamycin SV was made available in 1966 by the generosity of Lepetit SpA, Milan, Italy, and through the good offices of Professor P. Sensi, Director of the Research Laboratories of that company. The following data were supplied by the manufacturer.

lot no.	R/140
description	dark-red crystals
solubility	5 g dissolves in 5 ml of water at 25°C
water (Karl Fischer method)	12.73%
pH (5% solution in water)	6.55
sulfated ash	8.42%
phosphorus (as sodium phosphate)	0.015%
potency (as rifamycin SV free acid)	846 "μg"/mg

Since the manufacturer considered " the presence of the water *necessary* for a good stability of the product " it was proposed in 1966 to the WHO Expert Committee on Biological Standardization that this material should be distributed into ampoules at a relative humidity of approximately 30%, under which conditions no change in the content of water was expected to occur. The contents of the ampoules would then be allowed to equilibrate in an atmosphere of nitrogen, without drying, for several days before the ampoules were sealed. The Committee

[1] Authorization.

considered that, since the preparation would have a high content of water, its stability under these conditions should be examined before its establishment and requested the NIMR, London, to examine the material in this way.

In March 1967 the contents of the bottle were mixed by rotation for 2 days at room temperature (20°C). The powder was then distributed into approximately 2 500 ampoules (non-actinic glass) in a controlled atmosphere of 29% ± 2% relative humidity so that each ampoule contained approximately 100 mg of material. The unplugged ampoules were allowed to remain open at room temperature at this constant humidity for 2 days for equilibration to occur, and were then fitted with capillary plugs, filled with dry nitrogen, and equilibrated in an atmosphere of nitrogen at a pressure of 680 mm Hg for 3 days, after which they were refilled with dry nitrogen and sealed by fusion of the glass.

The moisture content of the material in the ampoules, determined as loss in weight after 5 h over phosphorus(V) oxide at 56°C and a pressure of less than 0.05 mm Hg, was found to be 8.55% w/w (mean of 19 ampoules). When the material in the ampoules was exposed to the atmosphere at a relative humidity of 57% it increased in weight by approximately 1% in 2 min and approximately 1.5% in 15 min.

Samples of the ampoules were stored at +56°C in order to assess stability. Assays of these samples gave the following results, indicating that the material had satisfactory stability.

Days at 56°C	*Percentage change of potency (mean of 2 ampoules)*
21	−0.6%
104	−4.3%
183	−7.25%

The material was established in 1967 as the International Reference Preparation of Rifamycin SV and, correcting for the change in water content, the International Unit was defined in 1967 as the activity contained in 0.001127 mg of the International Reference Preparation, corresponding to a potency of 887 IU/mg.

References

WHO Expert Committee on Biological Standardization (1966,[1] 1967, 1968).

RISTOCETIN AND RISTOCETIN B

A suitable sample of ristocetin was obtained in 1960 by the generosity of Abbott Laboratories, North Chicago, Ill., USA, through the good offices of Dr E. J. Matson of that company. The material, which was identical with the Abbott Laboratories working standard, was supplied with the following data.

weight	94 g in vials each containing 1 g
reference no.	C-SP-104
potency (without drying)	950 "μg"/mg
(potency has been established against primary standard PA-48-626A)	
ristocetin A content	98.0%
residue on ignition	0.14%
moisture	4.2%
heavy metals	2.5 ppm

After distribution into ampoules the moisture content of the material in the ampoules, measured as loss in weight at 56.5°C over phosphorus(V) oxide at a pressure of 0.01 mm Hg, was found to be 0.45% w/w. The potency of the material in the ampoules corrected for the change in moisture content was therefore 987 "μg"/mg. This material was established in 1960 as the International Reference Preparation of Ristocetin.

Since the ristocetin preparations used clinically are mixtures consisting predominantly of ristocetin A with ristocetin B in varying proportions, there was a need for two international reference preparations, one for ristocetin A and one for ristocetin B. The International Reference Preparation of Ristocetin was suitable to serve as an international reference material of ristocetin A.

A sample of ristocetin B, Lot 1466-80E (4.7 g) was obtained in 1963 by the generosity of Abbott Laboratories, North Chicago, Ill., USA, through the good offices of Dr W. W. Wright of the FDA. For distribution, the material was dissolved in water at a concentration of 5 mg/ml; the pH was adjusted to 4.25 with hydrochloric acid to ensure complete solution, and accurately measured aliquots of this solution were distributed into 890 ampoules,

[1] Authorization.

the volume of the aliquot being such that each ampoule contained 5.04 mg of ristocetin B preparation. Check weighings on 16 ampoules showed that the variation in size of the aliquots was $\pm 0.6\%$. After freeze-drying, the ampoules were treated by the standard procedure for drying and sealing.

Samples of the preparation of ristocetin B and the International Reference Preparation of Ristocetin were examined by electrophoresis for 4 h at 1 800 V and 80 mA, in agar gel with " tris–maleate " buffer (2-amino-2-(hydroxymethyl)-1,3-propanediol, maleic acid, and sodium hydroxide) at pH 7.0 by the method of Lightbown & de Rossi (1965). Three dosage levels of each preparation were used—namely, 4.0 mg/ml, 0.8 mg/ml, and 0.16 mg/ml, 4.0 μl of each being applied to the agar. After electrophoresis, the positions of the separated components were identified biologically by applying a layer of nutrient agar seeded with *Bacillus subtilis* and incubating at 37°C. Fig. 1 shows the results of such a separation. From the width of the zones of inhibition, the percentage of ristocetin A in the preparation of ristocetin B, and of ristocetin B in the International Reference Preparation of Ristocetin were calculated. The results of three experiments are shown in the following tabulation.

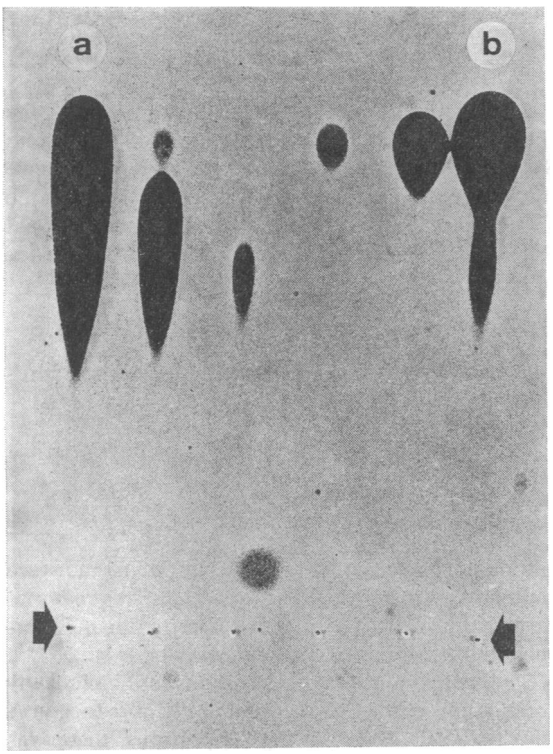

Fig. 1. Electrophoretic examination of international reference preparations of (a) ristocetin B and (b) ristocetin. Arrows indicate the origin. From left to right the amounts of ristocetin B (a) are 16 μg, 3.2 μg, and 0.64 μg, and of ristocetin (b) 0.64 μg, 3.2 μg, and 16 μg.

Experiment	*Percentage of ristocetin B in the International Reference Preparation of Ristocetin*	*Percentage of ristocetin A in the preparation of ristocetin B*
1	5.0	11.6
2	5.4	9.7
3	5.7	8.65
mean	5.3	9.9

The preparation of ristocetin B was considered to be satisfactory and was established (1964) as the International Reference Preparation of Ristocetin B.

For various reasons, this antibiotic is of limited availability and it was decided in 1965 that an international collaborative assay for the definition of an international unit was not necessary at that time.

References

Ristocetin: WHO Expert Committee on Biological Standardization (1959,[1] 1961, 1966); Ristocetin B: WHO Expert Committee on Biological Standardization (1964a,[1] 1964b, 1966).

SPIRAMYCIN

A suitable sample (100 g) of spiramycin base (reference no. JM 42) was obtained in 1962 by the generosity of May & Baker Ltd., Dagenham, England, and through the good offices of Mr F. J. Paxon of that company. The following analytical data were supplied by the manufacturer.

appearance	granular powder
colour	faintly yellow
odour	slight
$[\alpha]_D^{20}$	$-83°$

[1] Authorization.

10% solution in H_2SO_4	slightly dim
colour of solution	N/12 000 iodine
nitrogen (in dry material)	3.25%
water (Karl Fischer method)	<1.0%
sulfated ash	<0.10%
chlorides	<0.01%
biological assay	3 200 units/mg
composition:	
spiramycin 1	65%
spiramycin 2	20%
spiramycin 3	15%

After distribution into ampoules, the moisture content of the material, measured as loss in weight at over 56°C over phosphorus(V) oxide at a pressure of 0.02 mm Hg, was found to be 0.38% w/w (mean of 12 ampoules). The material in the ampoules increased in weight by approximately 1% w/w when exposed to the atmosphere for 30 min at a relative humidity of 45%.

This material was established in 1962 as the International Reference Preparation of Spiramycin and the International Unit was defined in 1964 as the activity contained in 0.0003125 mg of the International Reference Preparation, corresponding to a potency of 3 200 IU/mg.

Stability

Samples of the International Reference Preparation of Spiramycin lost 6% of activity after storage at 56°C for 4 years.

Note

The French and the Canadian national standards for spiramycin have been prepared from part of the identical batch of spiramycin that was used for the International Reference Preparation of Spiramycin.

References

WHO Expert Committee on Biological Standardization (1961, 1963,[1] 1964b).

TRIACETYLOLEANDOMYCIN

A sample (200 g, batch CR/1) of triacetyloleandomycin was received. It was obtained in 1962 by the generosity of Pfizer Ltd., Sandwich, England, as a possible British Standard and was found suitable for international use. The following information was supplied by the manufacturer.

biological potency	835 units/mg
$[\alpha]_D$ (200 mg in 10 ml of chloroethylene)	−18.6°
volatile substances	0.64%
heavy metals	10 ppm
iron	20 ppm
free from mono- and diacetyl oleandomycin when examined chromatographically	

After distribution into ampoules, the contents of 10 ampoules chosen at random were examined by heating at 56°C over phosphorus(V) oxide at a pressure of 0.02 mm Hg for 5 h. The mean loss of weight was 0.23% w/w. When samples of the material in open ampoules were exposed to the atmosphere at a relative humidity of 70%, they increased in weight by 0.3% w/w in 7 min and by 0.5% w/w in 37 min.

When assayed in the NIMR, London, against the triacetyloleandomycin preparation of the US National Formulary (American Pharmaceutical Association, 1965), a potency of 830 units/mg was obtained. The mean of this value, and the potency stated by the manufacturer, gave a value of 833 units/mg.

This material was established in 1962 as the International Reference Preparation of Triacetyloleandomycin and the International Unit was defined in 1962 as the activity contained in 0.00120 mg of the International Reference Preparation, corresponding to a potency of 833 IU/mg.

References

WHO Expert Committee on Biological Standardzation (1963).[1]

[1] Including the authorization.

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